The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains

Hassink, G.C.

CHAPTER 6 UBIQUITINATION OF MHC CLASS I HEAVY CHAINS IS ESSENTIAL FOR DISLOCATION BY HUMAN CYTOMEGALOVIRUS-ENCODED US2 BUT NOT BY US11

Gerco Hassink, Martine Barel, Sjaak van Voorden, Marjolein Kikkert, and Emmanuel Wiertz

Submitted

Abstract

The HCMV-encoded glycoproteins US2 and US11 target newly synthesized MHC class I heavy chains for degradation by mediating their dislocation from the ER back into the cytosol, where they are degraded by proteasomes. A functional ubiquitin system is required for US2- and US11-dependent dislocation of the class I heavy chains. It has been assumed that the class I heavy chain itself is ubiquitinated during the dislocation reaction. To test this hypothesis, all lysines within the class I heavy chain were substituted. The lysine-less class I molecules could no longer be dislocated by US2, despite the fact that the interaction between the two proteins was maintained. Interestingly, US11 was still capable of dislocating the lysine-less heavy chains into the cytosol. Ubiquitination does not necessarily require lysine residues, but can also occur at a protein's N-terminus. To investigate the potential role of N-terminal ubiquitination in heavy chain dislocation, a lysine-less ubiquitin moiety was fused to the N-terminus of the class I molecule. This lysine-less fusion protein was still dislocated in the presence of US11. Ubiquitination could not be detected *in vitro,* either for the lysine-less heavy chains, or for the lysine-less ubiquitin-heavy chain fusion protein.

Our data show that, even though dislocation of the lysine-less class I heavy chains requires a functional ubiquitin system, the heavy chain itself does not serve as the ubiquitin acceptor. This finding sheds new light on the role of the ubiquitin system in the dislocation process.

Introduction

A key step in the mammalian defense against viral infections is the presentation of virus-encoded peptides by MHC class I molecules to cytotoxic T-cells. The peptides presented by the class I molecules are frequently derived from newly synthesized proteins, degraded by proteasomes [1,2]. In case of a virus infection, a proportion of the peptides will be of viral origin. This system allows efficient detection and elimination of the infected cells by cytotoxic T cells. HCMV encodes two glycoproteins, US2 and US11, which cause rapid degradation of newly synthesized MHC class I heavy chains by mediating their retrograde transport or 'dislocation' from the ER into the cytosol, where they are degraded by proteasomes [3,4]. In this way, HCMV eludes the surveying cytotoxic T-cells.

The disposal of ER proteins via dislocation into the cytosol, followed by proteasomal degradation, is a common feature of all eukaryotic cells. The

US2- and US11-mediated degradation of MHC class I molecules has been instrumental in elucidating several mechanistic aspects of this pathway [3-16]. A wide variety of ER-luminal proteins are degraded through dislocation to the cytosol. They include mutated and/or misfolded proteins such as CFTRΔF508 and CPY*, oxidized proteins, and non-complexed, redundant subunits of multimeric complexes like TCRα chains (reviewed in [17-19], MHC class I heavy chains [20], and MHC class II α and β chains [21,22]. In addition, the expression of various proteins is regulated through dislocation and proteasomal degradation, e.g. HMG-CoA reductase (HMGR) and apo-lipoprotein B100 (reviewed in [17]).

How exactly ER proteins are exported to the cytosol is still poorly understood. It has been suggested that misfolded proteins are transported to the cytosol via the same channel through which they entered the ER, the Sec61 translocation complex [3,23-29]. Other potential constituents of a 'dislocon' are Derlin-1-3 and multi-membrane spanning E3 ligases such as HRD1, GP78, and TEB4 [16,30-33]. In the cytosol, glycoproteins are stripped of their N-linked glycans by an N-glycanase [34-37] and subsequently degraded by the proteasome in a ubiquitin-dependent manner.

A functional ubiquitin system is not only essential for the final proteasomal degradation, it has also been shown to be indispensable for the initial dislocation of ER proteins [7,29,38-46]. Dislocation of CPY* and HMGR, for example, depends on the ubiquitin ligase Hrd1p [29,41-43]. The mammalian ortholog of Hrd1p, HRD1, facilitates the dislocation of CD3δ and TCRα [45]. Furthermore, the US11-dependent dislocation of class I heavy chains [7,44], the dislocation of TCRα [38,40], as well as the dislocation of the μs chain of IgM [46] and IR_{322} [39] all depend on a functional ubiquitin system.

Initially, lysine residues within the dislocation substrate were considered to be the main target for ubiquitination, but it is becoming increasingly clear that this is not necessarily true in all cases. Replacement of all the lysines within the TCRα chain, or substitution of the lysines within the cytoplasmic tail of the MHC class I heavy chain by arginines does not affect the dislocation of these proteins [13,38,47]. The lysine residues within the cytoplasmic tail of MHC class I are also dispensible for the ubiquitin-dependent dislocation by the gamma herpesvirus 68-encoded Mk3 protein, a viral E3 ligase [48]. These findings are surprising, since the lysines within the cytoplasmic tail of these proteins would appear to be the most obvious targets for the cytosolic ubiquitin system. In other cases, however, cytoplasmic lysines are essential ubiquitination targets, as is illustrated by the HIV Vpu-induced dislocation of CD4, which depends on lysine residues within the cytosolic domain of CD4 [49]. The dislocation of the soluble ER-lumenal proteins CPY*

and RI_{332} also depends on the ubiquitinating system [39,50,51]. In those cases, luminal residues are only accessible to the ubiquitin ligation machinery after (partial) dislocation, implying that the intitial steps of the retrotranslocation reaction do not require ubiquitination of the substrate *per se*.

To acquire further insight into the role of the ubiquitin system in the retrograde transport of ER (glyco-)proteins, we utilized the extremely efficient dislocation of MHC class I heavy chains in the context of HCMV US2 and US11. In this model, the dislocation and the subsequent degradation of the heavy chains can be evaluated as two separate events, both temporally and spatially. We investigated whether the actual retrotranslocation requires the conjugation of ubiquitin to the target protein itself. To this end, a series of class I heavy chain mutants was constructed in which all lysine residues were substituted. To evaluate the possible contribution of the substrate's N-terminus, a lysine-less, non-cleavable ubiquitin moiety was fused to the N-terminus of the class I heavy chains. While the lysine-less class I molecules were no longer dislocated by US2, retrograde movement took place as normal in the presence of US11. Dislocation of the mutant heavy chains nevertheless required a functional ubiquitin system. While these results confirm the essential contribution of the ubiquitin system to glycoprotein dislocation, they indicate at the same time that retrotranslocation of the substrate can take place in the absence of ubiquitination of the substrate itself.

Results

US2-mediated dislocation of MHC class I heavy chains is ubiquitin-dependent

Previous studies have shown that the dislocation of MHC class I heavy chains by US11 requires a functional ubiquitin system [44]. It has become clear that US2 and US11-dependent dislocation of class I heavy chains differ in several ways. For example, US2 and US11 differ in their preference for HLA class I haplotypes and cytoplasmic tail composition of the heavy chains [52-54]. The fact that US11-mediated dislocation of class I heavy chains requires Derlin-1, but US2-mediated dislocation does not, indicates that the mechanism of the dislocation reaction is also different [14,15]. To investigate whether, like US11, US2-mediated dislocation requires a functional ubiquitin system, MHC class I heavy chains were co-expressed with US2 in TS20 cells, which have a temperature-sensitive mutation within the ubiquitin activating enzyme E1 [55]. Degradation of the class I heavy chains was compared at the permissive (33°C) and non-permissive temperature (40°C), in the presence of proteasome inhibitor. At the permissive temperature, dislocation of the class I heavy chains proceeded as normal (Fig. 1A, lanes 2-4). Removal of the single

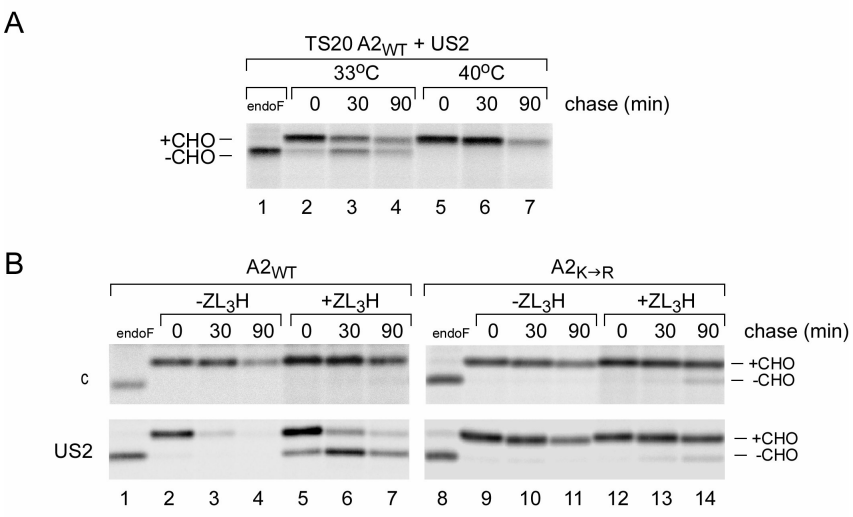
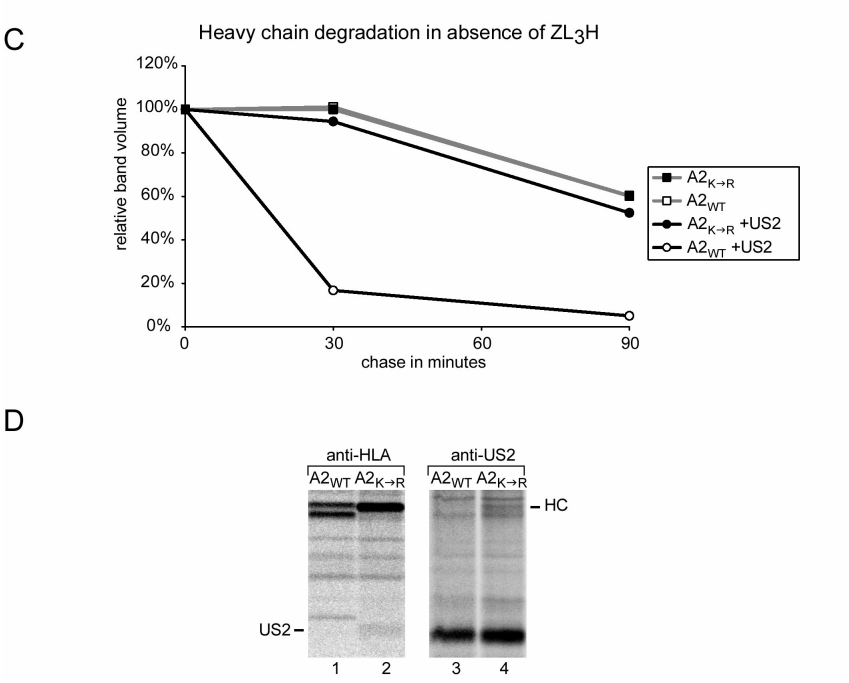


Figure 1. Dislocation of MHC class I molecules by US2 is dependent on the ubiquitin system and requires lysine residues within the class I heavy chains.
(A) First, TS20 cells expressing wild type HLA-A2 ($A2_{WT}$) and US2 were incubated at either 33°C or 40°C for 2 h. Next, the cells were starved, pulse-labeled with ^{35}S Met/Cys for 10 min, and chased for 30 and 90 min. The latter steps were all performed in the presence of proteasome inhibitor (ZL_3H), at the indicated temperatures. HLA-A2 molecules were immunoprecipitated with the MR24 anti-heavy chain antibody and separated on SDS-polyacrylamide gel (10 %). The presence or absence of carbohydrate side chains is indicated as +CHO or –CHO, respectively. The t=0 sample was treated with N-glycosidase F as described (EndoF; displayed in the first lane). (B) TS20 cells expressing either $A2_{WT}$ or $A2_{K\rightarrow R}$ were transduced with control (c) or US2-expressing retroviruses. Cells were pulse-labeled for 10 min and chased for 30 and 90 min at 33°C, with or without the proteasome inhibitor ZL_3H. MHC class I heavy chains were immunoprecipitated using the MR24 antiserum. Samples were displayed on SDS/PAGE (10 %). (C) Graph depicting the quantified results of the pulse-chase analysis shown in **A** (results obtained in the absence of proteasome inhibitor). (D) $A2_{WT}$ and $A2_{K\rightarrow R}$-expressing TS20-US2 cells were metabolically labeled for 60 min with ^{35}S Met/Cys in presence of the proteasome inhibitor ZL_3H. The cells were lysed in the presence of digitonin as described, and subjected to immunoprecipitations with either the MR24 anti-heavy chain antibody or an antibody directed against US2.

N-linked glycan from the class I heavy chains by cytosolic N-glycanase resulted in the emergence of a deglycosylated degradation intermediate (lanes 2-4). The latter migrates at the same level as class I heavy chains, obtained at the end of the pulse, treated with N-glycanase *in vitro* (lane 1). At the non-permissive temperature, the breakdown intermediate was not observed, indicating that dislocation of the class I heavy chains was inhibited (Fig. 1A, lanes 5-7). These results show that a functional ubiquitin system is essential for the dislocation of MHC class I heavy chains in the presence of US2.

Lysine-less MHC class I heavy chains are not dislocated by US2

MHC class I heavy chains carry a number of lysine residues that may



serve as acceptor sites for ubiquitin in the course of the dislocation and degradation process. To investigate whether these lysines are required for the ubiquitin-dependent dislocation by US2, we constructed a recombinant HLA A*0201 molecule, $A2_{K\rightarrow R}$, in which all 13 lysines were substituted for arginines. TS20 cells were transduced with retroviruses expressing the $A2_{K\rightarrow R}$ or $A2_{WT}$ heavy chains. In addition, the cells were transduced with a retrovirus expressing US2, or with a control virus. The stability of the wild type and recombinant class I heavy chains was evaluated in pulse-chase experiments (Fig. 1B). The $A2_{WT}$ heavy chains were relatively stable in the absence of US2 (Fig. 1A, upper-left panel, lanes 2-4). When US2 was expressed in these cells, the $A2_{WT}$ heavy chains were degraded rapidly (Fig. 1B, lower-left panel, lanes 2-4). In the presence of the proteasome inhibitor ZL_3H, a deglycosylated degradation intermediate appeared, migrating at the level of endoglycosidase F-treated heavy chains (compare lanes 1 and 5-7).

Like the $A2_{WT}$ heavy chains, the $A2_{K\rightarrow R}$ molecules were stable in cells not expressing US2 (Fig. 1B, upper-right panel). Contrary to the $A2_{WT}$ molecules, however, the $A2_{K\rightarrow R}$ heavy chains were not degraded in the presence of US2 (Fig. 1B, lower panels, compare lanes 2-4 and 9-11). A

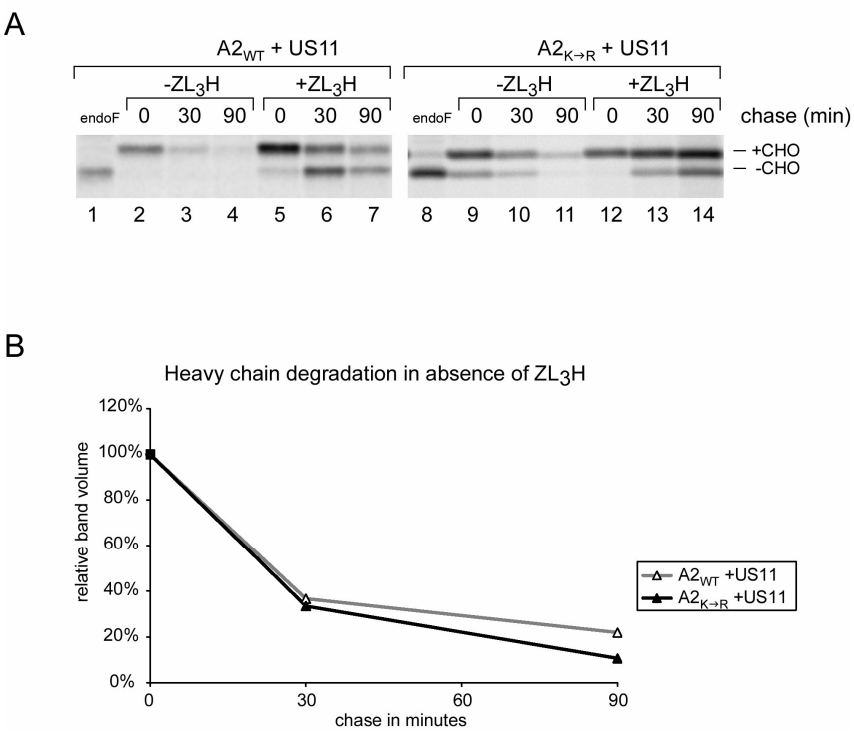


Figure 2. US11-mediated dislocation of MHC class I molecules does not depend on lysine residues within the class I heavy chains.

(A) US11-positive TS20 cells expressing either $A2_{WT}$ or $A2_{K\rightarrow R}$ were pulsed and chased as described in Fig. 1. MHC class I heavy chains were immunoprecipitated using the MR24 antiserum. Samples were analyzed on SDS/PAGE (10 %). (B) Quantification of the pulse-chase experiments performed in the absence of proteasome inhibitor (Fig. A, lanes 2-4 and 9-11; glycosylated heavy chains only).

quantification of the degradation of full-length heavy chains in the absence of proteasome inhibitor is shown in Fig. 1C. These results indicate that the lysine residues within MHC class I heavy chains are essential for the US2-mediated dislocation of these chains.

US2 can interact with lysine-less MHC class I heavy chains

The stabilization of the $A2_{K\rightarrow R}$ molecules in the presence of US2 might be the result of a lack of interaction of the lysine-less heavy chains with US2. Although the lysines had been replaced with arginines, which have very similar characteristics as far as side chain composition, structure and polarity are concerned, it cannot be excluded that these alterations affected the interaction with US2. Therefore, we investigated whether US2 was still capable of interacting with the lysine-less class I heavy chains in co-

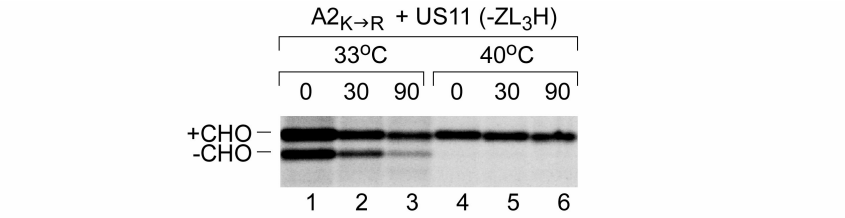


Figure 3. US11-mediated dislocation of $A2_{K\rightarrow R}$ is ubiquitin-dependent.
TS20 cells expressing $A2_{K\rightarrow R}$ and US11 were pre-incubated at either 33°C or 40°C for 2 h. Subsequently, the cells were starved, pulse-labeled with ^{35}S Met/Cys for 10 min, and chased for 30 and 90 min at the temperatures indicated. The experiment was performed in absence of proteasome inhibitor. MHC class I heavy chains were immunoprecipitated and displayed on SDS/PAGE (10 %).

immunoprecipitation experiments (Fig. 1D). Immunoprecipitation of the $A2_{K\rightarrow R}$ molecules resulted in co-precipitation of US2 (lane 2). Conversely, the anti-US2 antibody was able to co-precipitate $A2_{K\rightarrow R}$ heavy chains (lane 4). More US2/heavy chain material could be co-precipitated in the $A2_{K\rightarrow R}$ expressing cell line than in the $A2_{WT}$ cells. The latter was likely due to the fact that a substantial amount of $A2_{WT}$ heavy chains had been dislocated and, hence, occurred as free molecules in the cytosol. In conclusion, these data show that the lysine-less MHC class I heavy chains were not insensitive to US2-mediated degradation as a result of their inability to interact with the viral protein.

MHC class I heavy chains lacking lysine residues are dislocated by US11 in a ubiquitin-dependent manner

Next, we investigated whether the lysine-less class I heavy chains could be dislocated in the presence of US11. For this purpose, US11 was co-expressed with either $A2_{WT}$ or $A2_{K\rightarrow R}$ heavy chains in TS20 cells. Wild-type heavy chains were rapidly degraded in the US11-expressing cells (Fig. 2A, lanes 2-4). Interestingly, unlike US2, US11 was capable of inducing dislocation of the $A2_{K\rightarrow R}$ molecules (Fig. 2A, lanes 9-11; Fig. 2B). Deglycosylated heavy chains emerged in the US11-expressing cells in the absence of ZL_3H (lanes 8-11), indicating that proteasomal degradation of the dislocated $A2_{K\rightarrow R}$ molecules was inhibited. Note that in the presence of proteasome inhibitor, the dislocation became the rate-limiting step, resulting in delayed appearance of the deglycosylated degradation intermediate (compare conversion rates of glycosylated (upper band) to deglycosylated (lower band) heavy chains in lanes 9-11 and 12-14).

These results clearly show that, in contrast to US2, US11 can dislocate heavy chains that lack lysine residues. To investigate whether the dislocation of the lysine-less heavy chains requires a functional ubiquitin system

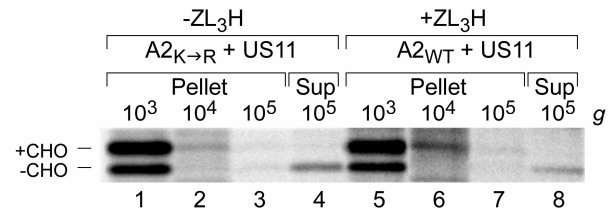


Figure 4. A soluble cytosolic $A2_{K\rightarrow R}$ degradation intermediate appears in US11-postive cells.
US11-positive TS20 cells expressing either $A2_{WT}$ or $A2_{K\rightarrow R}$, were labeled with ^{35}S Met/Cys for 30 minutes and subjected to subcellular fractionation using differential centrifugation as described. Pellet fractions were solubilized in NP40 lysis mix. MHC class I heavy chains were immunoprecipitated from the pellet fractions and the 10^5 *g* supernatant (Sup) and displayed on SDS/PAGE (10%). The experiments with the $A2_{WT}$-expressing cells, but not the $A2_{K\rightarrow R}$-expressing cells, were performed in the presence of ZL_3H.

nonetheless, we compared the degradation of the lysine-less class I molecules in the TS20 cells at 33°C and 40°C (Fig. 3). At 33°C, the $A2_{K\rightarrow R}$ molecules were dislocated, and, again, deglycosylated heavy chains emerged in the absence of proteasome inhibitor (lanes 1-3). At 40°C, the dislocation of lysine-less class I molecules was inhibited completely. These observations indicate that a functional ubiquitin system is essential for US11-dependent dislocation of lysine-less MHC class I heavy chains.

In the absence of proteasome inhibitor, a soluble degradation intermediate of $A2_{K\rightarrow R}$ can be observed in the cytosol of US11-expressing cells

The AAA ATPase p97-Ufd-Npl4 complex is required for membrane extraction of (partly) dislocated ER proteins that are still associated with the ER membrane. This final step might require ubiquitination of the substrate [8,9,39,46]. Deglycosylated lysine-less degradation intermediates of MHC class I molecules are detectable in the US11 cells (Fig. 2A and 3), but it is unknown if these are still associated with the ER membrane. To investigate whether these deglycosylated heavy chains had been fully released into the cytosol, a cell fractionation experiment was performed (Fig. 4). Note that the experiment with the $A2_{K\rightarrow R}$-expressing cells was performed in the absence of proteasome inhibitor. Deglycosylated $A2_{K\rightarrow R}$ and $A2_{WT}$ accumulated in the 10^5g supernatants representing the cytosolic fractions (Fig. 4, lanes 4 and 8), confirming that lysine-less class I heavy chains are indeed extracted from the ER membrane.

$A2_{K\rightarrow R}$ forms a complex with US11 and Derlin-1

Besides being dependent on the cytosolic p97 complex, US11-mediated dislocation of MHC class I heavy chains also requires the ER-resident membrane protein Derlin-1 [14,15]. US2-mediated dislocation, on the other

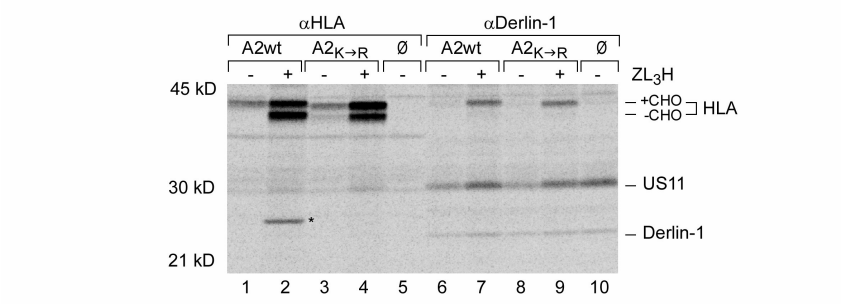


Figure 5. Derlin-1 interacts with $A2_{K\rightarrow R}$.
$A2_{WT}$, $A2_{K\rightarrow R}$-expressing, or HLA-negative TS20-US11 cells were pulse-labeled for 15 minutes with ^{35}S Met/Cys and chased for 30 minutes in the presence or absence of ZL_3H. Cells were lysed in digitonin-containing lysis buffer and subjected to immunoprecipitation with either the MR24 anti-heavy chain antibody or anti Derlin-1 antibody. * unidentified 25kD protein.

hand, does not depend on Derlin-1, although degradation of US2 itself does [15]. Derlin-1 is the human homologue of the yeast Der1p, which is required for the degradation of ER proteins in yeast, including misfolded CPY* [56]. To evaluate whether $A2_{K\rightarrow R}$ can also form a complex with Derlin-1, we investigated whether we could co-isolate $A2_{WT}$ and $A2_{K\rightarrow R}$ with Derlin-1 from digitonin lysates prepared from metabolically labeled cells. Fig. 5 shows that $A2_{WT}$ as well as $A2_{K\rightarrow R}$ molecules co-precipitated with Derlin-1 (lanes 7 and 9, respectively). Despite the presence of both intact and deglycosylated MHC class I heavy chains in the lysates (lanes 2 and 4), Derlin-1 selectively interacted with the glycosylated form of the class I molecules. Interestingly, US11 co-precipitated with Derlin-1 even in the absence of class I heavy chains, suggesting that US11 links Derlin-1 to the class I heavy chains.

In conclusion, these results indicate that lysine-less MHC class I heavy chains also associate with Derlin-1 in US11-expressing cells.

US11-mediated dislocation of MHC class I does not require N-terminal ubiquitination

Theoretically, the lysine-less class I heavy chain could still be ubiquitinated at its N-terminus. Therefore, the role of N-terminal ubiquitination was investigated in more detail using various ubiquitin-heavy chain fusion proteins. Either wild type ubiquitin or a lysine-less ubiquitin moiety was fused to the N-terminus of the class I heavy chains. Previously, it has been shown that US11-mediated dislocation of class I molecules requires polyubiquitination [13]. Attachment of lysine-less ubiquitin to the N-terminus of the class I heavy chain will prevent the formation of multi-ubiquitin chains at this site. The C-terminal glycine 76 of ubiquitin was replaced by a valine, creating a version that is no longer sensitive to deubiquitinating enzymes [57-60].

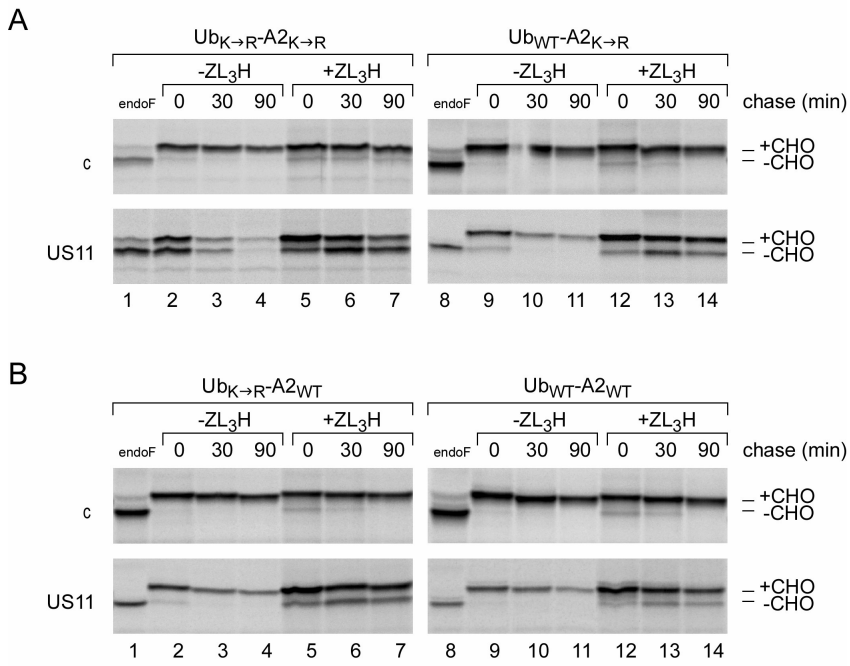
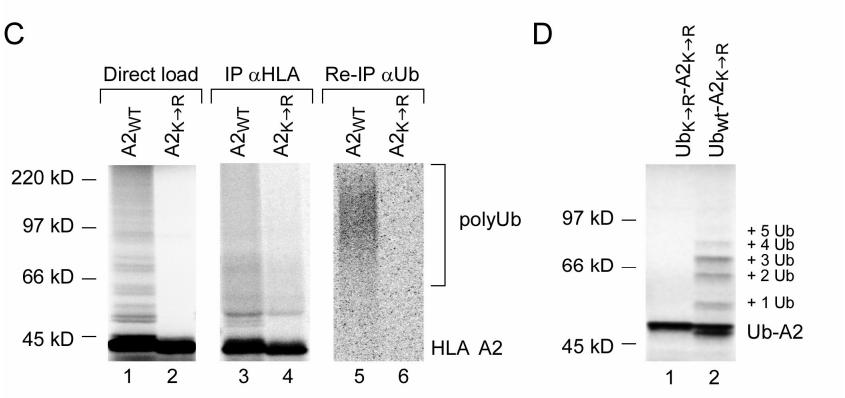


Figure 6. Dislocation of MHC class I-ubiquitin fusion proteins by US11.
(A) TS20 and TS20-US11 cells expressing $Ub_{K→R}$-$A2_{K→R}$ or Ub_{WT}-$A2_{K→R}$ fusion proteins were pulse-labeled for 10 min with ^{35}S Met/Cys and chased for 30 and 90 min at 33°C, with or without the proteasome inhibitor ZL_3H. HLA-A2 molecules with (+CHO) or without (-CHO) carbohydrate side chain were immunoprecipitated with the MR24 anti-heavy chain serum and analyzed on an SDS/polyacrylamide gel (10 %). The t=0 samples were treated with N-glycanase F (F). (A) Experiment as in (A) using cells expressing $Ub_{K→R}$-$A2_{WT}$ or Ub_{WT}-$A2_{WT}$ fusion proteins. (C) $A2_{WT}$ and $A2_{K→R}$ constructs were translated *in vitro* in the presence of ^{35}S-methionine in the absence of microsomes. One third of the translation product was loaded on gel directly (left panel). Two third of the translation product were subjected to immunoprecipitation with anti heavy chain antibody (MR24). One half of the immunoprecipitate was loaded on gel (middle panel), and the other half was subjected to a subsequent re-immunoprecipitation with the monoclonal antibody P4D1, recognizing polyubiquitin chains (right panel). (D) $Ub_{K→R}$-$A2_{K→R}$ and Ub_{WT}-$A2_{K→R}$ constructs were translated *in vitro* and loaded onto a 10 % SDS-polyacrylamide gel.

Four fusion proteins were constructed, consisting of ubiquitin (Ub) or lysine-less ubiquitin ($Ub_{K→R}$) fused with its C-terminus to the N-terminus of either wild type or lysine-less HLA-A2. The fusion proteins were stably expressed in TS20 cells using recombinant retroviruses.

In pulse chase experiments performed with US11-negative control cells, the $Ub_{K→R}$-$A2_{K→R}$ fusion protein remained stable throughout the chase (Fig. 6A, upper-left panel, lanes 2-4). In US11-expressing cells, $Ub_{K→R}$-$A2_{K→R}$ was dislocated and degraded (Fig. 6A, lower-left panel, lanes 2-4). This indicates that polyubiquitination at the N-terminus of the class I molecules is



not essential for US11-mediated dislocation. Dislocation of $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ resulted in the immediate emergence of the deglycosylated breakdown intermediate, despite the absence of proteasome inhibitor in the experiment (Fig. 6A, lanes 2-4). Apparently, like $A2_{K\rightarrow R}$, $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ can be dislocated into the cytosol, but is degraded less efficiently, resulting in a temporary accumulation of deglycosylated heavy chains.

Ub_{WT}-$A2_{K\rightarrow R}$, $Ub_{K\rightarrow R}$-$A2_{WT}$ and Ub_{WT}-$A2_{WT}$ were all dislocated and degraded in the presence of US11 (Fig. 6A, lanes 9-11; Fig. 6B). For the Ub_{WT}-$A2_{K\rightarrow R}$ fusion protein, very little deglycosylated class I was observed (Fig. 6A, lanes 9-11). This indicates that the N-terminal ubiquitin successfully created a ubiquitin fusion degradation (UFD) substrate [60,61]. In the absence of US11, all fusion proteins were stable throughout the chase, showing that ubiquitin fusion by itself did not induce dislocation of the class I heavy chains (Fig. 6A and B, upper panels).

The data presented so far are based on the assumption that $A2_{K\rightarrow R}$ and $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ cannot serve as ubiquitination substrates. To test this premise, ubiquitin conjugation to $A2_{K\rightarrow R}$ and $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ was evaluated *in vitro*. Translation of the MHC class I heavy chain constructs in the absence of microsomal membranes renders their hydrophobic transmembrane domains accessible to cytosolic chaperones and quality control mechanisms [18,19] thereby promoting their degradation through the ubiquitin-proteasome pathway. Fig. 6C, left panel, shows a direct load of translated wild type and lysine-less heavy chains. The wild-type construct produces an additional smear of high molecular weight material. Immunoprecipitation with an anti-MHC class I heavy chain antibody revealed that this smear contained class I molecules (middle panel). Subsequent re-immunoprecipitation with a poly-ubiquitin-specific antibody identified this material as ubiquitin-conjugated

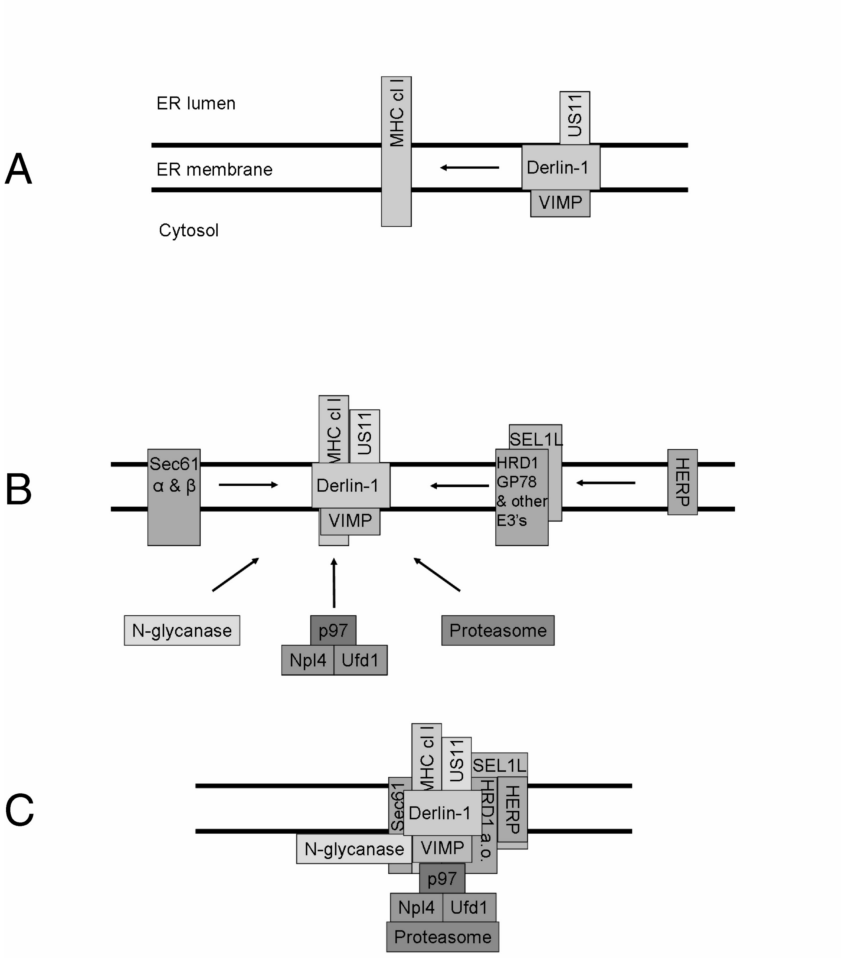


Figure 7. Formation of the dislocation complex mediating retrograde transport of MHC class I heavy chains in the context of HCMV US11.

class I heavy chains (right panel). Ubiquitinated heavy chains were not detectable for $A2_{K\rightarrow R}$ (Fig. 6C, lanes 2, 4 and 6).

Ubiquitination of $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ and Ub_{WT}-$A2_{K\rightarrow R}$ was evaluated in the experiment presented in Fig. 6D. Whereas translation of $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ did not yield ubiquitinated material, Ub_{WT}-$A2_{K\rightarrow R}$ resulted in a ladder of bands corresponding to heavy chains carrying one or more ubiquitin moieties. These experiments show that neither the lysine-less MHC class I heavy chains, nor the $Ub_{K\rightarrow R}$-$A2_{K\rightarrow R}$ fusion protein serve as ubiquitination substrates *in vitro*.

Altogether, these results indicate that dislocation of MHC class I heavy chains in the context of US11 does not require ubiquitination of the heavy chains themselves. However, ubiquitination promotes the degradation of dislocated heavy chains by proteasomes. Fusion of ubiquitin to the class I molecules does not accelerate their dislocation, supporting the conclusion that N-terminal ubiquitination does not play a role in US11-initiated extraction of heavy chains from the ER membrane. The finding that ubiquitinated $A2_{K\rightarrow R}$ cannot be detected *in vitro* suggests that the N-terminus is not a favorable ubiquitination site.

Discussion

In this study, we used the HCMV US2- and US11-mediated dislocation of MHC class I heavy chains as a model to investigate the role of the ubiquitin system in the dislocation and degradation of ER proteins. We show that the lysines within the class I molecule are essential for dislocation in the context of US2. In contrast, in US11-dependent dislocation, ubiquitination of the lysines or the N-terminus of the class I heavy chain does not play a role. US2 and US11, however, both require a functional E1 enzyme to effect dislocation of MHC class I molecules.

Although substitution of lysine residues within the MHC class I molecules does not prohibit their dislocation by US11, it does affect the efficiency of degradation once the heavy chains have reached the cytosol. As a result, deglycosylated heavy chains become visible in the cytosol in the absence of proteasome inhibitor (Figures 2A, 3 and 6). The breakdown intermediates appear early after synthesis. Inhibition of the catalytic activity of the proteasome results in a different pattern, with the deglycosylated heavy chains emerging at later time points during the chase (Fig. 2A). This difference in kinetics may be explained by the fact that the substitution of lysines does not block heavy chain dislocation, but mainly affects the degradation of dislocated molecules by the proteasome. In contrast, inhibition of the proteolytic activity of the proteasome seems to also cause a retardation of heavy chain dislocation, resulting in the delayed appearance of the heavy chains in the cytosol and accumulation at later time points. Thus, whereas heavy chain degradation is the rate-limiting step in case of lysine-less substrates, dislocation appears to be the rate-limiting step when the catalytic activity of the proteasome is blocked. It is interesting to note that proteasome inhibitors completely inhibit the retrograde movement of other ER dislocation substrates, including tyrosinase [62], ribophorin 332 [39], proparathyroid hormone related protein [63], TCRα [64], CD3δ [65,66], and MHC

class II α and β chains [21,22]. For those substrates, dislocation and proteasomal degradation may be more closely linked than for US11 mediated degradation of class I heavy chains.

Contrary to US11, US2 requires lysines within the MHC class I heavy chain for its dislocation into the cytosol. The stabilization of the $A2_{K\rightarrow R}$ molecules in the presence of US2 might be the result of a lack of interaction of the lysine-less heavy chains with US2. According to crystal structure data, two lysine residues occur within the US2 interaction region of the class I heavy chain. One of these, K_{176}, is directly in contact with US2 [67]. In the A2 mutants used in our study, however, the lysines were replaced with arginines, which have very similar characteristics as far as side chain composition, structure and polarity are concerned. Our data indicate that US2 can indeed still interact with the $A2_{K\rightarrow R}$ heavy chains. In addition, it has been shown that substitution of the lysine residues at position 127 and 144 of HLA-A2 does not affect the ability of US2 to retain this MHC class I mutant [68]. The question remains: how do lysines within the class I molecules contribute to US2-mediated dislocation? Previously, it has been shown that lysines within the cytoplasmic tail of class I heavy chains, and even the entire cytoplasmic tail, are dispensable for dislocation by US2 [47,52,53]. Combined, these findings suggest that ER-located lysine residues of the heavy chains play a role in dislocation by US2. Theoretically, US2 could induce a partial dislocation or 'prolapse' of part of the heavy chain into the cytosol, resulting in cytosolic deposition of luminal lysine residues. Although this possibility cannot be excluded, this scenario is unlikely. Alternatively, ubiquitin could serve as a ratcheting molecule during the extraction of the class I heavy chains. Previous studies have indicated that a ratcheting function of polyubiquitin alone is insufficient for dislocation of class I heavy chains by US11[13]. Whether this is also the case for US2-mediated retro-translocation of heavy chains remains to be established.

It has become clear that the dislocation of glycoproteins from the ER involves a wide array of proteins. The precise composition of the 'dislocation complex' is unclear as yet and could, in fact, be flexible, depending on which substrate has to be relocated to the cytosol. In addition to Derlin-1, VIMP, and p97, E3-ligases may also form a part of the dislocation complex [16,30,31]. In the case of MHC class I dislocation by US11, the reaction commences with the interaction of the viral glycoprotein with the newly synthesized class I heavy chain (Fig. 7). US11 links the class I heavy chain to the complex of Derlin-1 and the small membrane spanning molecule VIMP [14,15]. Interestingly, US11 also occurs in a complex with Derlin-1 in the absence of class I heavy chains (Fig. 5, lane 10), indicating that it is indeed US11 that targets Derlin-1 to the heavy chains. The next step might be the recruitment of translocon

subunits [3,23-29]and ER-resident ubiquitin ligase-complexes such as HERP-HRD1-SEL1L and GP78 [16,30,31]. The transmembrane regions of the multi-membrane spanning E3 enzymes may form a conduit around the class I heavy chain, possibly together with Derlin-1 and other membrane proteins, as has been hypothesized for the twin-arginine translocons occurring in peroxisomes and plant thylakoid membranes [69,70]. The advantage of forming a 'dislocon' after the substrate has been identified is that it will be adapted to the nature of the substrate [11,12]. Such an *ad hoc* arrangement would also allow the inclusion of transmembrane regions of substrates such as CFTRdelta508 and apolipoprotein B100 (reviewed in [17]). Finally, it is possible that the p97-Ufd1-Npl4 complex and the proteasome are attracted to, or even stabilize the dislocation complex [8,9]. Using the ATPase activity of p97 and/or the proteasome, the dislocation substrate may be extracted from the ER membrane. In the course of this process, the heavy chain is unfolded and deglycosylated by N-glycanase. The latter has been found to bind to Derlin-1 [37,71]. The fact that the dislocation reaction is dependent on a functional ubiquitin system, combined with the observation that US11-mediated dislocation of the MHC class I molecule does not involve ubiquitination of the class I heavy chain itself, suggests that the dislocation reaction requires ubiquitination of adaptor proteins that recruit other components into the dislocation complex. Thus, the ubiquitinated proteins may serve as a recognition signal for the p97-Ufd1-Npl4 complex and, possibly, for other components implicated in the retrograde movement of substrates into the cytosol.

Materials & Methods

Plasmids

Constructs expressing lysine-less MHC class I molecules were prepared by PCR using primers containing lysine to arginine codon mutations (see below); HLA A2*0201 was used as a template [44]. The following primers were used:

GAGTGGGCCCTCACTCTCCGTGTCTCC (antisense) and GGAGACACGGAGAGTGAGGGCCCACTC (sense) (bp 258-284), CCTCTCTCAGGGCGATGTAATCCCTGCCGTCG (antisense) and CGGCAGGGATTACATCGCCCTGAGAGAGG (sense) (bp 426-457), CGCCTCCCACCTGTGCCTGGTGGTC (antisense) and CAGACCAC CAGGCACAGGTGGGAGG (sense) (bp 493-519), TGCGTTCTGGG GGCGTCCGTGCGCTGCAGCGTCTCCCTCCCGTTC (antisense) and GAACGGGAGGGAGACGCTGCAGCGCACGGACGCCCCCAGAAC GCA (sense) (bp 591-635), ACCACAGCCGCCCACCTCTGGAAGG

(antisense) and GAACCTTCCAGAGGTGGGCGGCTG (sense) (bp 788-815), GGGTGAGGGGCCTGGGCAAACC (antisense) and AGGGTTTGCCCAGGCCCCTCAC (sense) (bp 863-886), CTCCTCTT CTATCTGAGCTCCTCCTCCTCCA (antisense) and GGAGGAGGAG CTCAGATAGAAGAGGAGGGAGCT (sense) (bp 994-130), and flanking sequences AGACCTCGAGCAGCTGTCTCACACTCTACAAGC (antisense) and TAATACGACTCACTATAGGGAGA (sense).

The PCR products were fused in three additional rounds of PCR. The resulting lysine-less MHC class I constructs were cloned into the pCR2 vector using the TOPO technology (Invitrogen) and subsequently subcloned into the pLZRS-EGFP vector (http://www.stanford.edu/group/nolan/). Ubiquitin-HLA fusion constructs were generated using a fusion PCR. Ubiquitin with the glycine at position 76 mutated to valine, and its lysine-less version [60](kindly provided by Dr. Nico Dantuma, Stockholm), were fused to wild-type or lysine-less HLA-A2. US2 [53] was subcloned into the pLZRS vector expressing an IRES element and truncated nerve growth factor receptor (ΔNGFR), using GATEWAY technology (Invitrogen).

Antibodies

MR24, a polyclonal antiserum directed against the luminal α3 domain of HLA-A2 [44], and the polyclonal anti-US11 and anti-HRD1 rabbit sera have been described previously [44,45]. Ubiquitin was detected using monoclonal antibody P4D1 (Santa Cruz). Anti-US2 rabbit serum [72] was kindly donated by Dr. T. Jones (Pearl River, NY). Anti-NGFR antibody was provided by Dr. M. Heemskerk (Leiden). Rabbit serum against Derlin-1[14] was kindly donated by Dr. T. Rapoport (Boston) and Dr. Y. Ye (Washington D.C.). The monoclonal antibody 20.4, directed against the nerve growth factor receptor (NGFR), was obtained from ATCC (#HB-8737).

Cells

The hamster cell lines E36 and E36ts20 (the latter referred to as TS20) [55] were maintained at 33°C in minimum essential medium (MEMα; Gibco BRL) supplemented with 10% (v/v) fetal calf serum (Greiner, Alphen a/d Rijn, The Netherlands), penicillin (100 units/ml) and streptomycin (100 μg/ml; Gibco BRL).

Retroviral transduction and FACS sorting

Replication-deficient recombinant retroviruses were produced using the pLZRS-EGFP or -ΔNGFR vectors and the Phoenix-A producer cells as described (http://www.stanford.edu/group/nolan/). Stable cell lines were generated by transduction of wild-type or US11-expressing TS20 cells [44] with

recombinant retrovirus encoding wild-type or mutant MHC class I molecules as well as EGFP. US2-expressing cells were generated by transduction of TS20 cells with retrovirus expressing US2 and ΔNGFR. A retrovirus encoding only EGFP served as a control. EGFP- and ΔNGFR-positive cells were sorted using a FACS Vantage cell sorter (Becton Dickinson). ΔNGFR-positive cells were stained with anti-NGFR antibody 20.4 (ATCC #HB-8737) and goat anti-mouse-TexasRed conjugate (Jackson, WA, U.S.A.).

Pulse–chase analysis, immunoprecipitation and SDS/PAGE

For metabolic labeling/pulse chase experiments, cells were starved in RPMI 1640 medium (Bio Whittaker) without methionine and cysteine at either 33°C or 40°C for approximately 1 h. For experiments performed at the non-permissive temperature, TS20 cells were pre-incubated at 40°C for 2-4 h, prior to the pulse-chase experiment. Starvation and pulse-chase incubations were all performed at the same temperature. Where indicated, proteasome inhibitor ZL_3H (Peptide Institute, Osaka, Japan) was used, added during starvation at a final concentration of 20 μM. The cells were metabolically labeled with 250 μCi of ^{35}S-labelled Redivue Promix (a mixture of L-[^{35}S]methionine and L-[^{35}S] cysteine) (Amersham) per 10^7 cells in starvation medium. For chase samples, radioactive medium was replaced with RPMI 1640 medium supplemented with 1 mM methionine and 0.1 mM cystine (Sigma).

The cells were lysed in Nonidet P-40 (NP40)-containing lysis buffer as described [44]. Immunoprecipitations were performed at 4°C on cleared lysates for 2-4 h using specific antiserum and Protein A Sepharose beads (Amersham, Roosendaal, The Netherlands). The beads were washed with NET buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5 % (v/v) NP-40) supplemented with 0.1 % SDS. The digitonin lysis buffer contained 1% (w/v) digitonin, 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 1 mM leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF; Roche, Woerden, The Netherlands). The washed beads were boiled in Laemmly sample buffer (40 mM Tris/HCl, pH 8.0, 4 mM EDTA, 8 %(w/v) SDS, 40 %(v/v) glycerol, 10 %(v/v) β-mercaptoethanol, and 0.1 % Bromophenol Blue) for 5 to 10 min. For re-immunoprecipitations, Laemmly samples containing 10 mM DTT were diluted with equal amounts of 10 mM iodo acetic acid (IAA) and subsequently diluted with NP40 lysis mix to a final volume of 1 ml. The samples were subjected to a sequential round of immunoprecipitation with the desired antibody. Samples were loaded onto SDS-polyacrylamide gels and exposed to a storage PhosphorImaging screen, which was scanned in a Personal Molecular Imager FX and analyzed with Quantity One software (Bio-Rad, Veenendaal, The Netherlands).

Subcellular fractionation

Fractionation of cells was performed essentially as described [4]. In brief, about 10^7 cells were starved and labeled for 30 min with ^{35}S-Redivue Promix as described above. Cells were washed and resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM potassium acetate, 1 mM EDTA, pH 7.6, supplemented with protease inhibitors leupeptin (0.1 mM) and AEBSF (10 mM)). Cells were placed on ice and homogenized using a Dounce homogenizer with a tight-fitting pestle (50 strokes). The homogenate was spun at 1,000*g* at 4°C in an Eppendorf centrifuge for 10 min. The pellet was saved and the supernatant was spun at 10,000*g* at 4°C for 30 min. The pellet was again saved and the supernatant spun at 100,000*g* at 4°C for 1 h. The latter two centrifugations were performed in a TLA 120.2 fixed-angle rotor, operated in a Beckman Optima2 TLX ultracentrifuge. All pellets were resuspended in NP-40 lysis buffer. HLA-A2 and US11 were immunoprecipitated simultaneously from the solubilized pellets and the 100,000*g* supernatant, and separated by SDS-PAGE.

In vitro transcription and translation and in vitro ubiquitination assays

HLA-A0201 in pCDNA3 or TOPO plasmids (Invitrogen) were linearized with XhoI and HindIII, respectively, and used for *in vitro* transcription with T7 polymerase (Promega). Transcripts were translated in the presence of L-[^{35}S]methionine in rabbit reticulocyte lysate without microsomes. Reaction mixtures of 25 µl were incubated at 30°C for 45 minutes. 16 µl samples were denatured by adding 34 µl PBS containing 1 % SDS and 10 mM DTT and boiling. at 95°C for 5 min. After the addition of 1 ml of non-denaturing buffer (1% Triton X-100, 50 mM Tris/HCl pH 7.5, 300 mM NaCl, 5mM EDTA, 0,02% sodium azide) and 10 mM IAA, immunoprecipitations were performed as described above.

Acknowledgements

We thank Hidde Ploegh, Brendan Lilley, Tom Rapoport, and Yihong Ye for generously providing reagents, and Kees Franke for constructing the GATEWAY vectors. This study was supported by grant RUL 1998-1791 from the Dutch Cancer Society (to GH)

References

1 Reits, E., Vos, J., Gromme, M. and Neefjes, J. (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature **404**, 774-778

2 Yewdell, J., Reits, E. and Neefjes, J. (2003) Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat.Rev.Immunol. **3**, 952-961

3 Wiertz, E., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T., Rapoport, T. and Ploegh, H. (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature **384**, 432-438

4 Wiertz, E., Jones, T., Sun, L., Bogyo, M., Geuze, H. and Ploegh, H. (1996) The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell **84**, 769-779

5 Tortorella, D., Story, C., Huppa, J., Wiertz, E., Jones, T., Bacik, I., Bennink, J., Yewdell, J. and Ploegh, H. (1998) Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. J.Cell Biol. **142**, 365-376

6 Shamu, C., Story, C., Rapoport, T. and Ploegh, H. (1999) The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. J.Cell Biol. **147**, 45-58

7 Shamu, C., Flierman, D., Ploegh, H., Rapoport, T. and Chau, V. (2001) Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. Mol.Biol.Cell **12**, 2546-2555

8 Rabinovich, E., Kerem, A., Frohlich, K., Diamant, N. and Bar-Nun, S. (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol.Cell Biol. **22**, 626-634

9 Ye, Y., Meyer, H. and Rapoport, T. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature **414**, 652-656

10 Furman, M., Ploegh, H. and Tortorella, D. (2002) Membrane-specific, host-derived factors are required for US2- and US11-mediated degradation of major histocompatibility complex class I molecules. J.Biol.Chem. **277**, 3258-3267

11 Fiebiger, E., Story, C., Ploegh, H. and Tortorella, D. (2002) Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. EMBO J. **21**, 1041-1053

12 Tirosh, B., Furman, M., Tortorella, D. and Ploegh, H. (2003) Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. J.Biol.Chem. **278**, 6664-6672

13 Flierman, D., Ye, Y., Dai, M., Chau, V. and Rapoport, T. (2003) Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retrotranslocation of proteins across the endoplasmic reticulum membrane. J.Biol.Chem. **278**, 34774-34782

14 Ye, Y., Shibata, Y., Yun, C., Ron, D. and Rapoport, T. (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature **429**, 841-847

15 Lilley, B. and Ploegh, H. (2004) A membrane protein required for dislocation of misfolded proteins from the ER. Nature **429**, 834-840

16 Lilley, B. and Ploegh, H. (2005) Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. Proc.Natl.Acad.Sci.U.S.A **102**, 14296-14301

17 Hampton, R. (2002) Proteolysis and sterol regulation. Annu.Rev.Cell Dev.Biol. **18**, 345-378

18 Sitia, R. and Braakman, I. (2003) Quality control in the endoplasmic reticulum protein factory. Nature **426**, 891-894

19 Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. Nat.Rev.Mol.Cell Biol. **4**, 181-191

20 Hughes, E., Hammond, C. and Cresswell, P. (1997) Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. Proc.Natl.Acad.Sci.U.S.A **94**, 1896-1901

21 Koppelman, B. and Cresswell, P. (1990) Rapid nonlysosomal degradation of assembled HLA class II glycoproteins incorporating a mutant DR alpha-chain. J.Immunol. **145**, 2730-2736

22 Cotner, T. and Pious, D. (1995) HLA-DR beta chains enter into an aggregated complex containing GRP-78/BiP prior to their degradation by the pre-Golgi degradative pathway. J.Biol.Chem. **270**, 2379-2386

23 Koopmann, J., Albring, J., Huter, E., Bulbuc, N., Spee, P., Neefjes, J., Hammerling, G. and Momburg, F. (2000) Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. Immunity. **13**, 117-127

24 Pilon, M., Romisch, K., Quach, D. and Schekman, R. (1998) Sec61p serves multiple roles in secretory precursor binding and translocation into the endoplasmic reticulum membrane. Mol.Biol.Cell **9** , 3455-3473

25 Sommer, T. and Jentsch, S. (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature **365**, 176-179

26 Gillece, P., Pilon, M. and Romisch, K. (2000) The protein translocation channel mediates glycopeptide export across the endoplasmic reticulum membrane. Proc.Natl.Acad.Sci.U.S.A **97**, 4609-4614

27 Plemper, R., Egner, R., Kuchler, K. and Wolf, D. (1998) Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. J.Biol.Chem. **273**, 32848-32856

28 Chen, Y., Le Caherec, F. and Chuck, S. (1998) Calnexin and other factors that alter translocation affect the rapid binding of ubiquitin to apoB in the Sec61 complex. J.Biol.Chem. **273**, 11887-11894

29 Bordallo, J., Plemper, R., Finger, A. and Wolf, D. (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol.Biol.Cell **9**, 209-222

30 Ye, Y., Shibata, Y., Kikkert, M., Van Voorden, S., Wiertz, E. and Rapoport, T. (2005) Inaugural Article: Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proc.Natl.Acad.Sci.U.S.A **102**, 14132-14138

31 Schulze, A., Standera, S., Buerger, E., Kikkert, M., Van Voorden, S., Wiertz, E., Koning, F., Kloetzel, P. and Seeger, M. (2005) The Ubiquitin-domain Protein HERP forms a Complex with Components of the Endoplasmic Reticulum Associated Degradation Pathway. J.Mol.Biol. **354**, 1021-1027

32 Hassink, G., Kikkert, M., Van Voorden, S., Lee, S., Spaapen, R., van Laar, T., Coleman, C., Bartee, E., Fruh, K., Chau, V. and Wiertz, E. (2005) TEB4 is a C4HC3 RING finger-containing ubiquitin ligase of the endoplasmic reticulum. Biochem.J. **388**, 647-655

33 Kreft, S., Wang, L. and Hochstrasser, M. (2006) Membrane Topology of the Yeast Endoplasmic Reticulum-localized Ubiquitin Ligase Doa10 and Comparison with Its Human Ortholog TEB4 (MARCH-VI). J.Biol.Chem. **281**, 4646-4653

34 Park, H., Suzuki, T. and Lennarz, W. (2001) Identification of proteins that interact with mammalian peptide:N-glycanase and implicate this hydrolase in the proteasome-dependent pathway for protein degradation. Proc.Natl.Acad.Sci.U.S.A **98**, 11163-11168

35 Hirsch, C., Blom, D. and Ploegh, H. (2003) A role for N-glycanase in the cytosolic turnover of glycoproteins. EMBO J. **22**, 1036-1046

36 Katiyar, S., Li, G. and Lennarz, W. (2004) A complex between peptide:N-glycanase and two proteasome-linked proteins suggests a mechanism for the degradation of misfolded glycoproteins. Proc.Natl.Acad.Sci.U.S.A **101**, 13774-13779

37 Katiyar, S., Joshi, S. and Lennarz, W. (2005) The retrotranslocation protein Derlin-1 binds peptide:N-glycanase to the endoplasmic reticulum. Mol.Biol.Cell **16**, 4584-4594

38 Yu, H., Kaung, G., Kobayashi, S. and Kopito, R. (1997) Cytosolic degradation of T-cell receptor alpha chains by the proteasome. J.Biol.Chem. **272**, 20800-20804

39 de Virgilio, M., Weninger, H. and Ivessa, N. (1998) Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. J.Biol.Chem. **273**, 9734-9743

40 Yu, H. and Kopito, R. (1999) The role of multiubiquitination in dislocation and degradation of the alpha subunit of the T cell antigen receptor. J.Biol.Chem. **274**, 36852-36858

41 Gardner, R. and Hampton, R. (1999) A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. J.Biol.Chem. **274**, 31671-31678

42 Gardner, R., Swarbrick, G., Bays, N., Cronin, S., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. (2000) Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J.Cell Biol. **151**, 69-82

43 Gardner, R., Shearer, A. and Hampton, R. (2001) In vivo action of the HRD ubiquitin ligase complex: mechanisms of endoplasmic reticulum quality control and sterol regulation. Mol.Cell Biol. **21** , 4276-4291

44 Kikkert, M., Hassink, G., Barel, M., Hirsch, C., van der Wal, F. and Wiertz, E. (2001) Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. Biochem.J. **358**, 369-377

45 Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., Van Voorden, S., Thanedar, S., Roitelman, J., Chau, V. and Wiertz, E. (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J.Biol.Chem. **279**, 3525-3534

46 Elkabetz, Y., Shapira, I., Rabinovich, E. and Bar-Nun, S. (2004) Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound p97/Cdc48p and proteasome. J.Biol.Chem. **279**, 3980-3989

47 Furman, M., Loureiro, J., Ploegh, H. and Tortorella, D. (2003) Ubiquitinylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum. J.Biol.Chem. **278**, 34804-34811

48 Wang, X., Connors, R., Harris, M., Hansen, T. and Lybarger, L. (2005) Requirements for the selective degradation of endoplasmic reticulum-resident major histocompatibility complex class I proteins by the viral immune evasion molecule mK3. J.Virol. **79**, 4099-4108

49 Schubert, U., Anton, L., Bacik, I., Cox, J., Bour, S., Bennink, J., Orlowski, M., Strebel, K. and Yewdell, J. (1998) CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. J.Virol. **72**, 2280-2288

50 Biederer, T., Volkwein, C. and Sommer, T. (1996) Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. EMBO J. **15**, 2069-2076

51 Biederer, T., Volkwein, C. and Sommer, T. (1997) Role of Cue1p in ubiquitination and degradation at the ER surface. Science **278**, 1806-1809

52 Gewurz, B., Wang, E., Tortorella, D., Schust, D. and Ploegh, H. (2001) Human cytomegalovirus US2 endoplasmic reticulum-lumenal domain dictates association with major histocompatibility complex class I in a locus-specific manner. J.Virol. **75**, 5197-5204

53 Barel, M., Ressing, M., Pizzato, N., van Leeuwen, D., Le Bouteiller, P., Lenfant, F. and Wiertz, E. (2003) Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation. J.Immunol. **171**, 6757-6765

54 Barel, M., Pizzato, N., van Leeuwen, D., Bouteiller, P., Wiertz, E. and Lenfant, F. (2003) Amino acid composition of alpha1/alpha2 domains and cytoplasmic tail of MHC class I molecules determine their susceptibility to human cytomegalovirus US11-mediated down-regulation. Eur.J.Immunol. **33**, 1707-1716

55 Kulka, R., Raboy, B., Schuster, R., Parag, H., Diamond, G., Ciechanover, A. and Marcus, M. (1988) A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1. J.Biol.Chem. **263**, 15726-15731

56 Knop, M., Hauser, N. and Wolf, D. (1996) N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. Yeast **12**, 1229-1238

57 Baker, R. (1996) Protein expression using ubiquitin fusion and cleavage. Curr.Opin.Biotechnol. **7**, 541-546

58 Varshavsky, A. (1996) The N-end rule: functions, mysteries, uses. Proc.Natl.Acad.Sci.U.S.A **93**, 12142-12149

59 Hondred, D., Walker, J., Mathews, D. and Vierstra, R. (1999) Use of ubiquitin fusions to augment protein expression in transgenic plants. Plant Physiol **119**, 713-724

60 Dantuma, N., Lindsten, K., Glas, R., Jellne, M. and Masucci, M. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat.Biotechnol. **18**, 538-543

61 Bachmair, A., Finley, D. and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. Science **234**, 179-186

62 Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V. and Hebert, D. (1997) Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc.Natl.Acad.Sci.U.S.A **94**, 6210-6215

63 Meerovitch, K., Wing, S. and Goltzman, D. (1998) Proparathyroid hormone-related protein is associated with the chaperone protein BiP and undergoes proteasome-mediated degradation. J.Biol.Chem. **273**, 21025-21030

64 Yang, M., Omura, S., Bonifacino, J. and Weissman, A. (1998) Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. J.Exp.Med. **187**, 835-846

65 Tiwari, S. and Weissman, A. (2001) Endoplasmic reticulum (ER)-associated degradation of T cell receptor subunits. Involvement of ER-associated ubiquitin-conjugating enzymes (E2s). J.Biol.Chem. **276**, 16193-16200

66 Menendez-Benito, V., Verhoef, L., Masucci, M. and Dantuma, N. (2005) Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. Hum.Mol.Genet. **14**, 2787-2799

67 Gewurz, B., Gaudet, R., Tortorella, D., Wang, E., Ploegh, H. and Wiley, D. (2001) Antigen presentation subverted: Structure of the human cytomegalovirus protein US2 bound to the class I molecule HLA-A2. Proc.Natl.Acad.Sci.U.S.A **98**, 6794-6799

68 Thilo, C., Berglund, P., Applequist, S., Yewdell, J., Ljunggren, H. and Achour, A. (2006) Dissection of the interaction of the human cytomegalovirus-derived US2 protein with MHC class I molecules: Prominent role of a single arginine residue in HLA-A2. J.Biol.Chem. **in press**

69 Schnell, D. and Hebert, D. (2003) Protein translocons: multifunctional mediators of protein translocation across membranes. Cell **112**, 491-505

70 Mori, H. and Cline, K. (2002) A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid [Delta]pH/Tat translocase. J.Cell Biol. **157**, 205-210

71 Suzuki, T., Park, H., Till, E. and Lennarz, W. (2001) The PUB domain: a putative protein-protein interaction domain implicated in the ubiquitin-proteasome pathway. Biochem.Biophys.Res.Commun. **287**, 1083-1087

72 Jones, T., Hanson, L., Sun, L., Slater, J., Stenberg, R. and Campbell, A. (1995) Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. J.Virol. **69**, 4830-4841